Dynamics of phosphorothioate oligonucleotides in normal and laser photocoagulated retina

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Abstract

***Aims*—To investigate the distribution, persistence, and stability of fluorescently labelled phosphorothioate oligonucleotides (PS-ODNs) in normal and laser photocoagulated retina following intravitreal injection in the rat.**

***Methods*—Fluorescently labelled PS-ODNs were injected intravitreally into pigmented eyes at doses of 0.5–10.0 nmol in 2.0 µl solution. The dynamics of PS-ODNs was evaluated by fluorescent microscopy of cryosections and flat mounted retinal pigment epithelium (RPE)-choroid-sclera. Genescan analysis was used to assess the integrity of PS-ODNs in the retina after injection. The dynamics of PS-ODNs was also evaluated in the retina following krypton laser photocoagulation with a protocol producing choroidal neovascularisation (CNV).**

***Results*—Following intravitreal injection the PS-ODNs demonstrated dose and time dependent distribution and persistence in the retina, where they accessed all neural layers. However, they preferentially accumulated in the RPE layer, demonstrated as bright granules in the cytoplasm of the cells. Injections of 5.0 and 7.5 nmol of PS-ODNs exhibited strong fluorescence in the retina for 6 weeks after injection. Genescan analysis demonstrated that the PS-ODNs remained almost completely intact for at least 12 weeks. Following laser treatment, the PS-ODNs were concentrated in the regions of laser photocoagulation and retained high intensity for at least 8 weeks after injection, particularly localised to macrophages, RPE, and the local choroidal tissue.**

***Conclusions*—These results indicate that PS-ODNs are stable and accessible to most neural layers of the retina, and they preferentially accumulate in the RPE layer following intravitreal injection. The successful delivery of PS-ODNs into normal and laser photocoagulated retina suggests that PS-ODNs may have potential in the development of therapy for attenuating retinal degenerations and CNV.**

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Antisense oligodeoxynucleotides (ODNs) designed to hybridise to specific genes have been used to inhibit the synthesis of cellular protein and may serve as potential therapeutic agents by selective inhibition of gene expression.[1–5] These compounds can penetrate across the cell membrane and, after specific hybridisation to mRNA, trigger translation arrest or degradation of the target molecule, leading to inhibition of biosynthesis of a specific protein. One of the initial obstacles in the use of ODNs in biological systems was the marked sensitivity of native phosphodiester ODNs to degradation by serum and cellular endonucleases and exonucleases, which has precluded the use of phosphodiester ODNs.[6] The poor stability of ODNs has been improved recently by a variety of chemical modifications of the backbone of ODNs, one of which produces phosphorothioate oligonucleotides (PS-ODNs) that have shown more resistance to degradation and advanced farthest as a class of potential clinical drug candidates.[7 8]

As one part of the central nervous system, the retina consists of neural cells including ganglion, amacrine, bipolar, horizontal, and photoreceptor cells. Neurodegeneration is a hallmark of many ocular diseases including glaucoma,[9] ischaemia initiated retinal neurodegeneration,[10] retinitis pigmentosa,[11] accidental laser induced retinal injury,[12] and macular degeneration such as age related macular degeneration (ARMD).[13 14] Recent investigations indicate that some genetic programmes may drive the progress of retinal degeneration by pathogenic genes.[9 11 15 16] The effectiveness of ODNs in affecting specific gene expression may open new avenues to rescue or attenuate retinal degeneration and other retinal diseases.[2–4 17 18]

Intravitreal injection is an established method of drug delivery for the treatment of retina-vitreous diseases. One of the advantages of intravitreal injection is the even access of the compounds to the retina. However, the fate of PS-ODNs in the retina following intravitreal injection varies according to different investigators and the animal species.[19–22] This study was designed to investigate further details of distribution, persistence, and stability of PS-ODNs in the rat retina by tracking fluorescently labelled random or vascular endothelial growth factor (VEGF) specific sequence PS-ODNs after intravitreal injection. These dynamics of PS-ODNs in the retina were also studied following laser photocoagulation.

Materials and methods

PS-ODNS SYNTHESIS AND THEIR LABELLING

PS-ODNs were synthesised on an ABI Model 392 DNA synthesiser (ABI, Foster City, CA, USA) using the phosphoramidite method according to the manufacturer's instructions. Two oligonucleotides, DS 012 (5' AGGAC-

Table 1 Dose dependent distribution of phosphorothioate oligodeoxynucleotide (DS 012) in the rat retina after 2 weeks' intravitreal injection

Doses (nmol)	*Eyes*	*GCL*	*INL*	*ONL*	*RPE*
10	4	++++	++++	+++	++++
5.0	4	++++	++++	++	++++
2.0	4	+++	+++	++	+++
1.0	4	++	+	+	+++
0.5	4	+	+	−	+++

GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; RPE = retinal pigment epithelium. The fluorescent signal was graded by the density and patterns of distribution. − = no signal; + = weak or moderate but scattered; ++ = moderate and evenly distributed; +++ = strong and evenly distributed; ++++ = very strong and evenly distributed.

Table 2 Time dependent distribution of phosphorothioate oligodeoxynucleotide (DS 012, 5.0 nmol) in the rat retina following intravitreal injection

Time (weeks)	*Eyes*	*GCL*	*INL*	*ONL*	*RPE*
1	4	++++	++++	+++	++++
2	3	++++	++++	++	++++
4	3	++	++	+	+++
6	3	++	++	+	+++
8	3	+	−	−	++

GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; RPE = retinal pigment epithelium. The fluorescent signal was graded by the density and patterns of distribution. − = no signal; + = weak or moderate but scattered; ++ = moderate and evenly distributed; +++ = strong and evenly distributed; ++++ = very strong and evenly distributed.

CTGTCAATTCCGGTG 3'), a randomly selected DNA sequence with no known match to any sequence in the genome database, and DS 013 (5' TCGCGCTCCCTCTCCG-GCTC 3'), a targeted sequence complementary to a region upstream of the translation initiation codon of human and rat universal versions of VEGF gene[20] were synthesised for this experiment. A fluorescent molecule, 6 carboxyfluorescein amidite (6-FAM: ABI, Foster City, CA, USA), was conjugated to the 5' terminal nucleotide according to the manufacturer's instructions to determine histological location using fluorescent microscopy and for Genescan analysis to assess oligonucelotide size and integrity. The concentrations of PS-ODNs were determined by optical density at 260 nm.

INTRAVITREAL INJECTION OF PS-ODNS IN NORMAL EYES

Normal pigmented rats (RCS/rdy p+) were anaesthetised and their pupils dilated as described previously.[23] The conjunctiva was cut and the sclera exposed. A 30 gauge needle was used to make an initial puncture of the sclera. Through this hole a 32 gauge needle attached to a 5 µl Hamilton syringe was passed into the vitreous cavity. The advancement of the needle was directly observed under an operating microscope when the needle tip lay in the vitreous cavity. Two µl of DS 012 (0.5, 1.0, 2.0, 5.0, 7.5, or 10.0 nmol) or DS 013 (7.5 nmol) diluted in PBS was delivered into the vitreous cavity. The needle was kept in the vitreous cavity for about 1 minute then withdrawn gently and antibiotic ointment was applied to prevent infection. The injected eyes were observed at 3 days after injection through an indirect ophthalmoscope to assess any sign of lens injury, retinal detachment, vitreous/retinal haemorrhage, or intraocular infection. As controls, 2 µl of 6-FAM (10, 100 nmol), sodium fluorescein (5.0, 500 nmol), or phosphate buffered saline (PBS) were injected as described above. The non-fluorescent parent 6-FAM molecule was chemically converted into the fluorescent form, representing the equivalent form of that attached to the PS-ODNs. This was injected at the same concentration and 10-fold higher than that conjugated with the PS-ODNs**.** All animal experiments adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

PS-ODNS INJECTION FOLLOWING KRYPTON LASER PHOTOCOAGULATION

Krypton laser irradiation (647.1 nm, Coherent Radiation System, CA, USA) was delivered through a Zeiss slit lamp with a hand held coverslide serving as a contact lens. Fifteen to 25 laser burns were applied in each eye around the posterior pole using our protocol which produces CNV.[23] Following laser photocoagulation, 2.0 µl of DS 012 (5.0 nmol) was injected intravitreally on the next day. The injected eyes were enucleated at 1, 2, 4, 6, and 8 weeks after injection. The sampled eyes were either snap frozen in liquid nitrogen or flat mounted onto glass slides and examined by fluorescent microscopy as described below.

FLUORESCENT MICROSCOPY AND CONFOCAL LASER SCANNING MICROSCOPY

The animals injected with different doses of fluorescently labelled DS 012 were killed at 2 weeks after injection (n=4 at each dose). The rats injected with DS 012 (5.0, 7.5 nmol), DS 013 (7.5 nmol), or vehicles (6-FAM, sodium fluorescein and PBS) in the normal eyes, and those injected with DS 012 (5.0 nmol) following laser photocoagulation, were killed at 1, 2, 4, 6, and 8 weeks after injection (n=3–4 at each time point). In the case of 6-FAM, eyes were also enucleated at 1 and 24 hours after injection (10, 100 nmol, n=2 each time point). The enucleated eyes were freshly embedded in OCT compound and snap frozen in liquid nitrogen without fixation. Cryosections of 12–14 µm thickness were cut with a Jung Frigocut 2800N Cryostat (Leica, Germany) and examined immediately by fluorescent microscopy. To confirm the intracellular localisation of fluorescently labelled PS-ODN, some sections were examined by a confocal laser scanning microscope as we described previously.[19]

PATHOGENICITY AND IMMUNOSTAINING FOR CD68

The eyes injected at doses of 0.5–10.0 nmol of DS 012, 2 weeks after injection, were counterstained with haemotoxylin for the evaluation of retinal morphology, and those injected with 10.0 nmol of DS 012 were used for immunohistochemistry of CD68 to detect whether the PS-ODNs induce ocular inflammation. The enucleated eyes were embedded in OCT and cut as mentioned above. The cryosections were mounted onto coated slides (2% 3-aminoproply-triethoxysilane, Sigma, A-3648) and stored at −70°C until use. A mouse monoclonal anti-rat CD68 antibody (Serotec Ltd, Kidlington, Oxford) was diluted 1:500 in 0.1 M PBS pH 7.4 containing 1% bovine serum albu-

min (BSA). Sections were fixed by 2% paraformaldehyde solution for 5 minutes and given 3 × 5 minute washes in PBS. The incubation was carried out in a moist chamber. Following incubation with the primary antibodies at 4°C overnight, sections were washed and incubated with Universal Dako Lasb2 (Dako, CA, USA, code K0609) biotinylated secondary antibody for 30 minutes. After 3 × 5 minute washes in PBS, sections were treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase, then incubated with streptavidin peroxidase for 30 minutes. Peroxidase was developed with 3-amino-9-ethylcarbarzol (AEC, Sigma, code A-6926) for 10 minutes to yield a red reaction product.

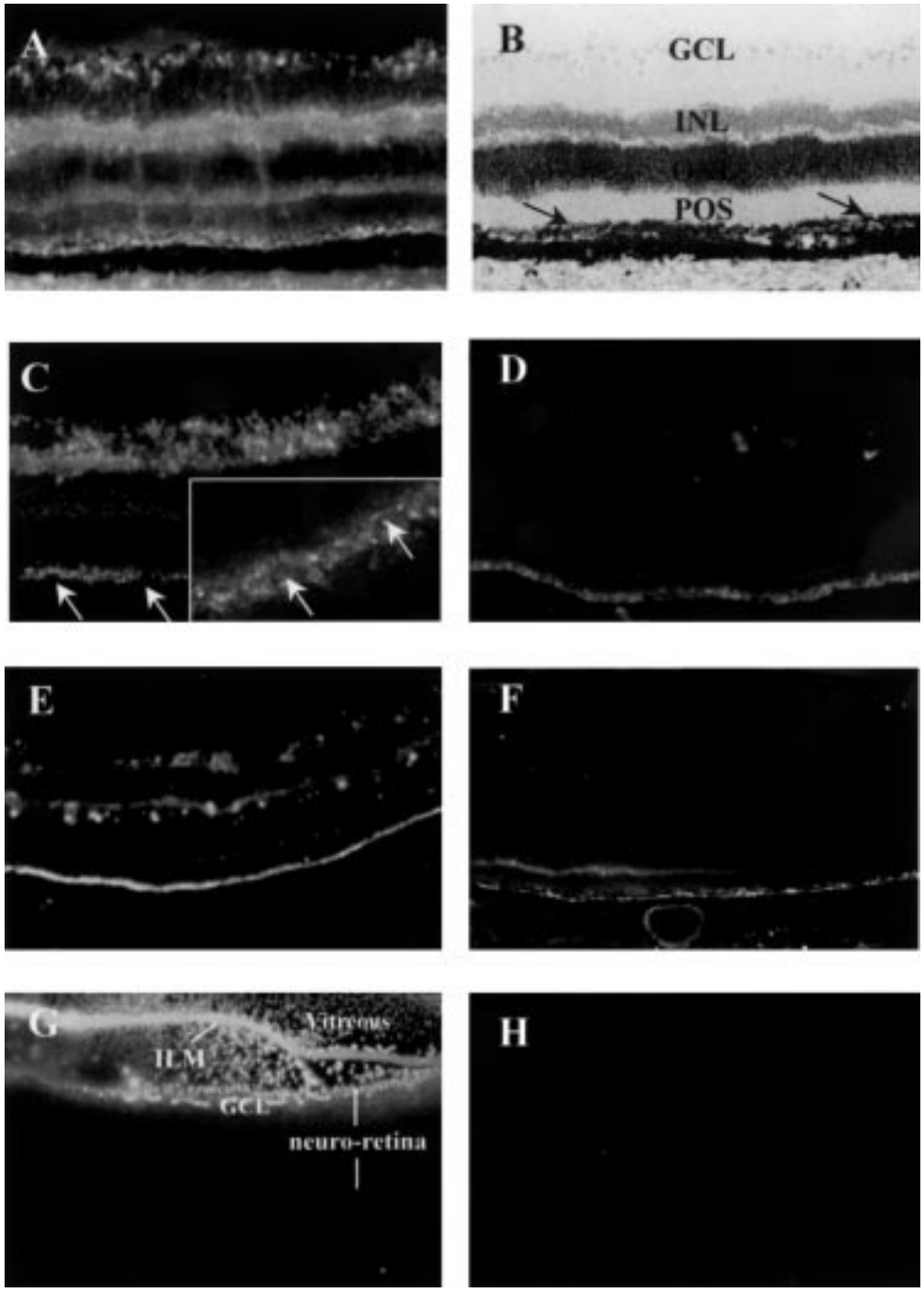

Figure 1 Distribution and persistence of PS-ODNs (DS 012) in the retina 2 weeks after intravitreal injection (A–D). (A) 10.0 nmol, (B) retinal morphology of (A), counterstaining with haemotoxylin. The arrows indicate the RPE layer. (C) 2.0 nmol, the arrows are pointing to the nuclei of RPE cells. Inset: cytoplasmic distribution of PS-ODN in RPE observed by confocal laser scanning microscopy. (D) 0.5 nmol. (E and F) 5.0 nmol, 6 weeks (E) and 8 weeks (F) after injection. (G and H) Fluorescent microscopic images from the control eyes injected with fluorescent 6-FAM at 1 hour (G) and 24 (H) hours after injection. The fluorescent 6-FAM was mostly confined to the vitreous but some just entered into the ganglion cell layer at 1 hour after injection (G). However, no fluorescent signal was detected in the neural retina at 24 hours after injection (H). Original magnifications: (A–H) ×50; inset of (C) ×100. RPE = retinal pigment epithelium; POS = photoreceptor outer segment; ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer; ILM = inner limiting membrane.

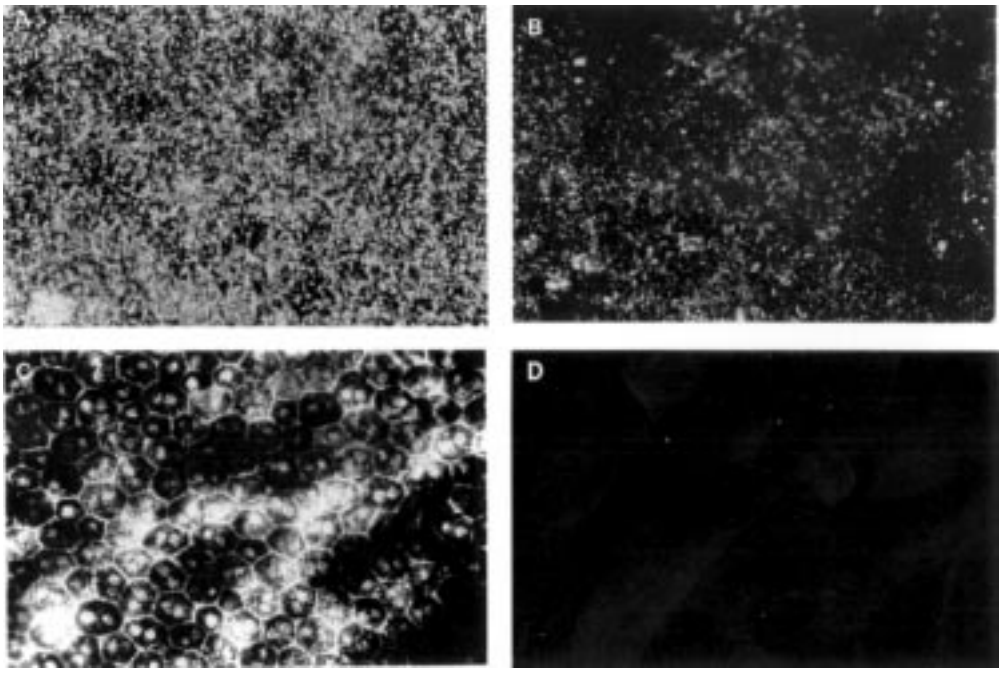

Figure 2 Distribution of fluorescently labelled PS-ODN (DS 012, 5.0 nmol) in the RPE cells by fluorescent microscopy of flat mounted RPE-choroid-sclera. (A) Two weeks after injection, the fluorescently labelled PS-ODNs appear as bright granules in the cytoplasm of the RPE cells. (B) Eight weeks after injection. (C) Microphotograph of the hexagonal RPE cells in (A), observed by light microscopy. (D) A control eye injected with 5.0 nmol of sodium fluorescein, 2 week after injection. Original magnification (A–D) ×100.

GENESCAN ANALYSIS

Genescan is an electrophoresis procedure that detects fluorescently labelled DNA and separates DNA on the basis of length. Three eyes injected with 7.5 nmol of DS 012 for 6, 8, and 12 weeks respectively were examined by a Genescan protocol modified to detect oligonucleotide sized DNA, to assess the integrity and persistence of PS-ODNs in the retina following intravitreal injection. ODN injected eyes and their contralateral non-injected control eyes were enucleated and dissected into retina and RPE-choroid-sclera complex. DNA was extracted from these fresh tissues using 200 µl of digestion buffer (50 mM TRIS HCl, pH 7.4, 0.5 $CaCl_2$, 1% SDS) containing 0.25 mg/ml proteinase K and mixed gently for 1 hour at 37°C. Solid residue was removed by centrifugation and the supernatant retained. The aqueous phase was extracted three times with phenol chloroform and the nucleic acid precipitated with ethanol and retained as a pellet.

DNA pellets from the eyes taken 6 weeks after injection were resuspended in 500 µl of deionised formamide. A 1.0 µl aliquot was removed then added to 1.0 µl of a size standard (TAM-500, ABI, Foster City, CA, USA, diluted 1 in 20 to observe the primer peak within the sensitivity range of instrument) and 12.0 µl of formamide, then denatured by heating at 95°C and quenching on ice. Samples were run on the ABI 310 Genetic Analyser using the POP 4 polymer (ABI, Foster City, CA, USA), where samples were injected for 5 seconds at 15 kV and electrophoresed at 15 kV for 20 minutes at 60°C using the ABI Prism 310 collection 1.0.2 and Genescan 2.1 software.

Samples at 8 and 12 weeks after injection were diluted differently as fluorescent microscopy had indicated a reduction of fluorescent signal. These samples were run using POP6 polymer to improve the resolution. The samples were precipitated and resuspended in 50 µl water, then run on the Genescan instrument by taking 13.0 µl of samples and 1.0 µl of internal size standard. Therefore, these samples were 130 times more concentrated than the 6 week samples. To ensure that the extracted ODNs were identical to the original form, the original DS 012 stored at −20°C was also run under the same conditions. Fluorescent 6-FAM run on Genescan was not detected. Sample run conditions using POP6 polymer required injection of samples for 5 seconds at 15 kV and electrophoresis at 7.5 kV for 65 minutes at 50°C using the same software.

A series of varying length ODNs (15-mer, 17-mer, 19-mer, 21-mer, and 25-mer), around the size of the injected ODN (20-mer), were synthesised with 6-FAM conjugated to the 5' terminal nucleotide. Approximately 1 pmol of each standard oligonucleotide was added to 1.0 µl internal size standard and 12 µl formamide and run under the similar conditions except using 2.5 kV injection voltage. These size standards were investigated to ensure that the modified run variables were capable of resolving ODN sized within this range and to ensure that the fluorescently labelled ODNs extracted from the injected eyes had undergone no nucleotide degradation.

FLAT MOUNTS OF RETINA AND RPE-CHOROID-SCLERA AND FLUORESCENT MICROSCOPY

The eyes injected with 5.0 nmol DS 012, with or without laser photocoagulation, were harvested

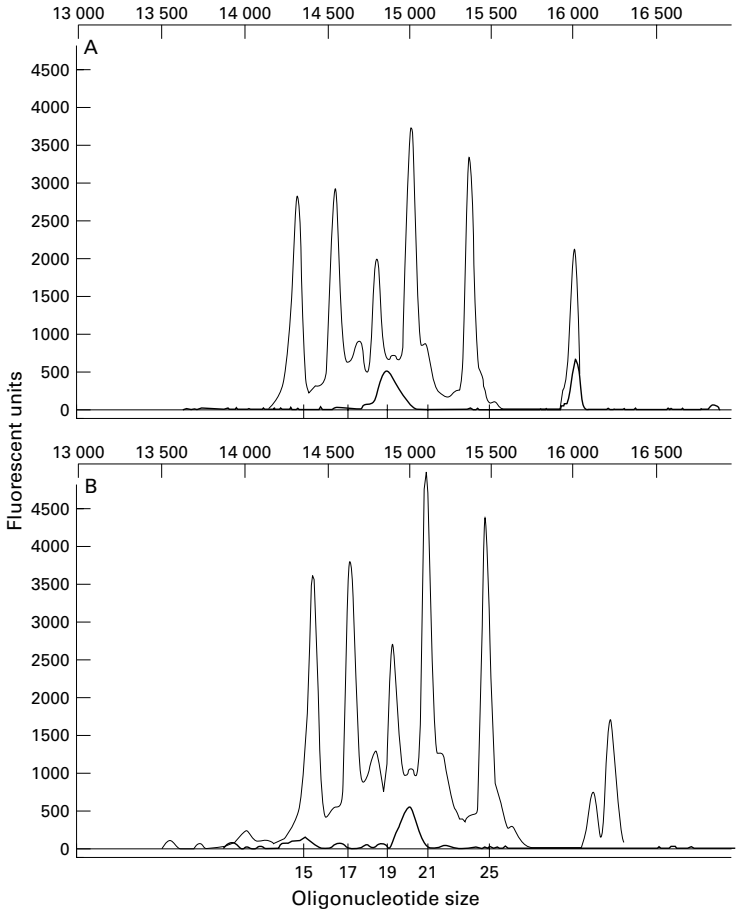

Figure 3 Accurate Genescan sizing of injected and uninjected PS-ODN using oligonucleotide size standards. (A) The original 20-mer DS 012 was compared directly with a series of PS-ODNs varying in size from 15 to 25 nucleotides in length and their electrophoretograms superimposed using the internal standard as a reference. Oligonucleotides within this range of size were clearly resolved using the Genescan analysis software under the altered run conditions. (B) DNA extracted from the retina of an eye 12 weeks following injection with DS 012 for 12 weeks was run and compared directly with the oligonucleotide size standard. The extracted oligonucleotide demonstrated some minor peaks representing N-1 degradation products.

at 1, 2, 4, 6, 8, weeks after injection for flat preparations (n=2–3 at each time point). The enucleated eyes were fixed with 2% paraformaldehyde for 30 minutes at 4°C. After washing in PBS, the eyes were cut via pars plana, and the anterior segment and lens removed. The eye cup was cut peripherally in four or five places and flat mounted on a glass slide under a dissecting microscope. The neural retina was gently removed and mounted onto the same slide using a fine forceps. Care was taken to avoid damaging the retina and RPE cells, particularly at the optic nerve head and in the area with laser burn spots. For the eyes injected with 5.0 nmol DS 012 following laser photocoagulation, the retina remained attached to the RPE-choroid-sclera for the flat mount preparation. The flattened samples were mounted with glycerol medium (Pittsburgh, PA, USA) and examined by fluorescent microscopy.

Results

DISTRIBUTION, PERSISTENCE, AND PATHOGENICITY OF PS-ODNS IN NORMAL RETINA

Intravitreal delivery of fluorescently labelled DS 012, 5.0, 7.5, and 10.0 nmol, resulted in a high intensity of fluorescence across the retina at 2 weeks after injection. The fluorescent signal was demonstrated throughout all neural layers including ganglion cell, inner nuclear and outer nuclear layers, and the RPE cells (Fig 1A). The eyes injected with lower doses of DS 012, 0.5, 1.0, and 2.0 nmol, showed weaker fluorescence in the retina (Fig 1C, D, Table 1). The persistence of PS-ODNs in the retina was also time dependent (Table 2). In the eyes injected with DS 012, 5.0, and 7.5 nmol, time matched photographic exposures indicated that the intensity of fluorescent signal remained strong for as long as 6 weeks after injection (Fig 1E). After 6 weeks the fluorescent signal reduced rapidly throughout the retina, and little signal was detected at 8 and 12 weeks after injection (Fig 1F). However, the PS-ODNs seemed to be preferentially accumulated in the RPE cells, as the RPE layer demonstrated a higher intensity of fluorescence relative to other cellular layers, particularly in the eyes injected with low doses of PS-ODNs or after a long time of injection with high doses of PS-ODNs (Tables 1, 2; Fig 1C, D, E). In the control eyes injected with 6-FAM, the fluorescent 6-FAM diffused from the vitreous cavity onto the retinal surface at 1 hour after injection (Fig 1G). However, no fluorescent signal was detected in the retina at 24 hours after injection in the eyes injected with either 6-FAM, sodium fluorescein or PBS alone (Fig 1H).

Antisense VEGF oligonucleotide (DS 013) did not show any difference from the random PS-ODN (DS 012) in distribution and persistence following intravitreal injection (data not shown).

The retinal morphology of the eyes injected with 10.0 nmol of DS 012, the highest dose tested in this experiment, was normal when observed by light microscopy (Fig 1B). However, a few infiltrating cells were detected at the site of injection in the eyes following PS-ODNs delivery, which was also observed in the eyes injected with 6-FAM or sodium fluorescein. These infiltrating cells were confirmed to be macrophages, as evidenced by positive staining for CD68. Other parts of the retina did not demonstrate any staining for CD68 following intravitreal injection (data not shown).

DISTRIBUTION OF PS-ODNS IN THE RPE

Examination of cryosections by fluorescent microscopy indicated that the PS-ODNs in the RPE cells seemed to localise to the cytoplasm, as the nuclei of the RPE cells appeared as dark spots (Fig 1C, arrows). The PS-ODNs in the cytoplasm of the RPE cells were clearly demonstrated by examination of cryosections with confocal laser scanning microscopy which shows the distinguished nuclei (Fig 1C, inset, arrows) of the RPE cells and the cytoplasm engorged with fluorescent granules. By examination of flat mounted RPE-choroid-sclera complex, the fluorescently labelled PS-ODNs were demonstrated as bright granules in the cytoplasm of the RPE cells (Fig 2A). However, the fluorescent signal in the RPE cells reduced dramatically at 8 weeks after injection (Fig 2B). With flat mounted RPE-choroid-sclera

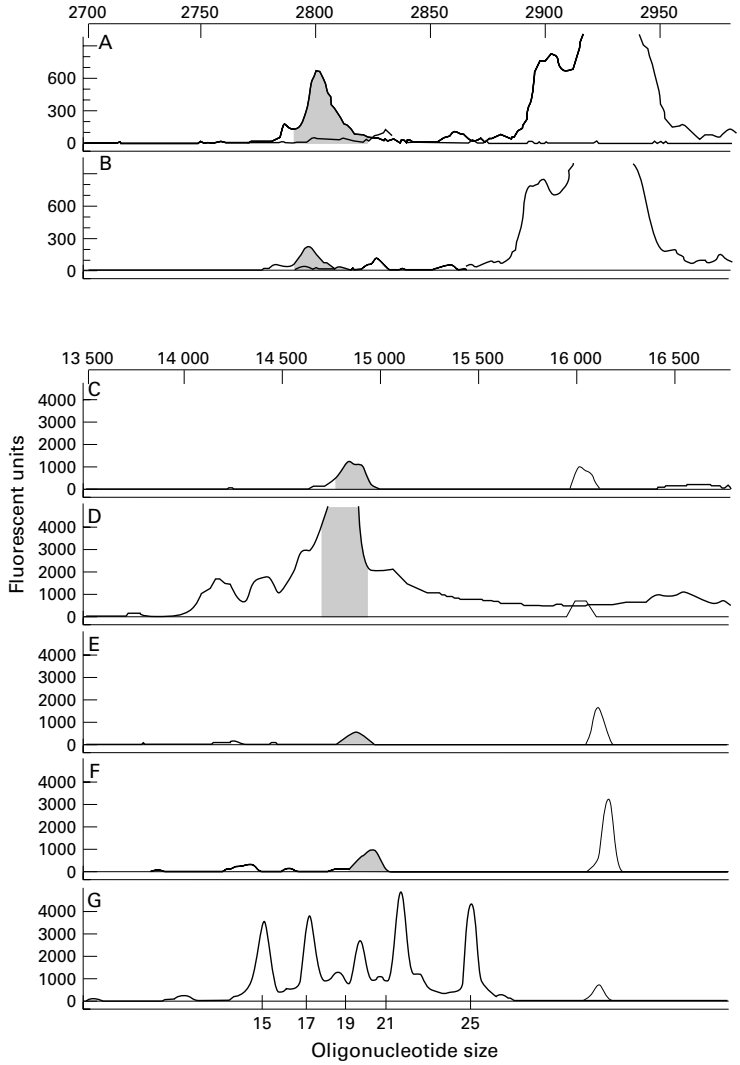

Figure 4 Persistence of PS-ODN following intravitreal injection demonstrated by Genescan analysis. Electrophoretograms of DNA extracted from the retina (A, C, and E) and the RPE-choroid-sclera complex (B, D, and F) at 6 weeks (A, B), 8 weeks (C, D), and 12 weeks (E, F) after injection with DS 012, and the oligonucleotide size standard (G). The internal standard is superimposed in all electrophoretograms at the same data collection point and the peaks for the PS-ODN are equivalent for both tissue samples at all time points. Note the actual fluorescent intensity of 6 week samples is effectively 130 times higher than that of 8 and 12 week samples.

sheets, the hexagonal morphology of RPE cells was recognised very well by light microscopy (Fig 2C). With time matched exposures, no fluorescent signal was observed in flat mounts of RPE-choroid-sclera following injection of 6-FAM or sodium fluorescein (Fig 2D).

STABILITY OF PS-ODNS FOLLOWING INTRAVITREAL INJECTION—GENESCAN ANALYSIS

Genescan electrophoretograms of the original DS 012 (non-injected, stored at −20°C before Genescan analysis) and DNA extracted from eye tissues were overlaid by aligning the internal size standard peak which was run simultaneously with the samples (Fig 3A and B). The size standards (15–25-mer) were resolved as clearly defined peaks indicating that the modified Genescan protocol is capable of resolving small variations in oligonucleotide length. This provided an accurate sizing of 20 nucleotides for both the original uninjected 20-mer DS012 (Fig 3A) and the ODN extracted from the retina at 12 weeks after injection (Fig 3B). The results showed that this system was able to resolve single nucleotide differences in PS-ODN length, and that the majority of the ODN extracted from eyes at 12 weeks after injection was full length with only minor amounts of N-1 degradation products.

For each set of samples (6, 8, and 12 weeks tissue extracts) the major peak was consistently detected at the same data collection point, indicating that DS 012 remained almost completely intact in the retina for up to 12 weeks following intravitreal injection (Fig 4). The relative comparison of fluorescent intensity, bearing in mind that the 6 week samples were effectively 130 times higher than that of 8 and 12 week samples, demonstrated that there were high levels of PS-ODNs in the neural retina and RPE-choroid-sclera at 6 weeks after injection (Fig 4 A, B). By 8 and 12 weeks after injection, the signals in the retina and RPE-choroid-sclera remained detectable but at considerably lower levels (Fig 4C–F). Degradation products down to 14 nucleotides were observed at 8 and 12 weeks (Fig 4D and F) which indicates that exonuclease activity at the 3' end of the ODN is occurring. This was most evident in the RPE-choroid-sclera than in the retina. It is estimated from comparison with the size standards of known concentration that the instrument can detect down to the order of 10 fmol/µl of ODN in the tissue extracted solution.

DISTRIBUTION OF PS-ODNS IN THE RETINA FOLLOWING KRYPTON LASER PHOTOCOAGULATION

Following krypton laser photocoagulation, the fluorescently labelled PS-ODN was found to be concentrated in the regions of laser photocoagulation, demonstrated as clumps of bright granules at the sites of laser spots, whereas untreated regions showed a diffuse and even distribution (Fig 5A). By 8 weeks (termination of this experiment), the regions of laser photocoagulation still retained a high fluorescent signal (Fig 5B).

By fluorescent microscopy, the fluorescent signal was demonstrated to be localised to infiltrating cells and the RPE within or adjacent to the regions of laser photocoagulation (Fig 5C, D). The local choroidal tissue also demonstrated some fluorescent signal (Fig 5C, D), which was usually absent in the choroid layer of normal eyes following intravitreal injection of PS-ODNs (Fig 1A, C–F). Most neural layers of the retina retained strong fluorescence at 4 weeks after injection (Fig 5C), consistent with what we have previously shown. By light microscopy, a large amount of pigment laden cells accumulated at the site of laser photocoagulation (Fig 5E). Subsequent immunohistochemistry further confirmed that most of them were macrophages, as evidenced by positive staining for CD68, a specific cellular marker for macrophages (Fig 5F). As macrophages are autofluorescent in fresh tissues, the eyes injected with 6-FAM or sodium fluorescein following laser photocoagulation were used as controls where only a small

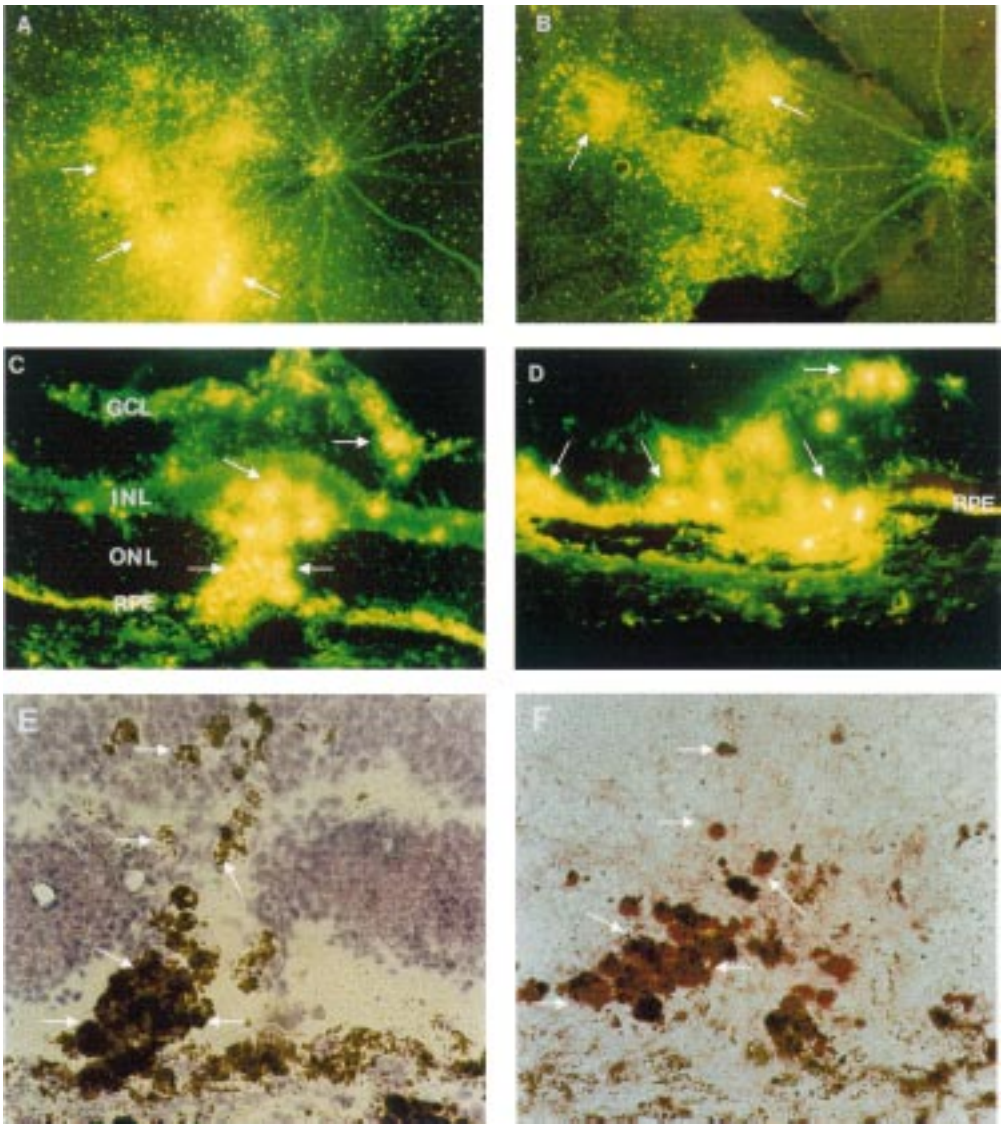

Figure 5 Distribution of PS-ODNs (DS 012, 5.0 nmol) in the retina following krypton laser photocoagulation. (A, B) Retinal whole mounts, 4 weeks (A) and 8 weeks (B) after injection; the PS-ODNs are concentrated in the regions of laser photocoagulation with an appearance of clumps of bright granules at the sites of laser burns (arrows). (C, D) Cryosections from eyes injected with DS 012 following krypton laser photocoagulation, 4 weeks (C) and 8 weeks (D) after injection. The fluorescent signal is particularly localised to infiltrating cells (arrows) and the RPE layer. (E) Higher magnification of (C) observed by light microscopy, counterstaining with haemotoxylin, the arrows indicating pigment laden infiltrating cells. (F) A serial section of (C) and (E), immunostaining for CD68 (arrows). Original magnification: (A, B) ×10; (C, D) ×50; (E, F) ×100. GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; RPE = retinal pigment epithelium.

number of very weakly fluorescent cells were observed at the site of laser photocoagulation (data not shown).

Discussion

In the present study, we used fluorescently labelled ODNs to monitor their distribution, persistence, and stability in the retina following intravitreal injection. Decrease or disappearance of fluorescence is thought to reflect their persistence and stability. To overcome the poor stability of phosphodiester ODNs, we synthesised nuclease resistant phosphorothioate ODNs (PS-ODNs). Although there is evidence that high doses of PS-ODNs may exert toxicity,[2 24 25] no retinal toxicity was observed in this study of rat eyes injected with 0.5–10.0 nmol of PS-ODNs. There was minimal cell infiltration at the site of injection. These infiltrating cells were attributed to mechanical penetration as they were also present in 6-FAM, sodium fluorescein and PBS injected eyes.

Intravenous administration of PS-ODNs results in very poor distribution in the central nervous system (CNS) including the eye.[26] Presumably, this is in large part due to poor penetration of ODNs through the blood-brain and blood-retina barriers. Thus, the dynamics of PS-ODNs following local microinjection is particularly important for their therapeutic applications in various CNS diseases. It has been reported that PS-ODNs can be safely delivered to wide areas of rat brain by microinjection. However, the injected PS-ODN was retained in situ for less than 48 hours.[27 28] The reason for quick elimination of PS-ODNs from the brain is not understood but may be related

to the most abundant blood supply, active metabolism, and high expression of multifunctional DNA repair enzymes.[29]

The fate of PS-ODNs in the retina varies according to different investigators and animal species following intravitreal injection.[19–22] In the present study, intravitreal injection of PS-ODNs, 5.0, 7.5, and 10.0 nmol, resulted in high fluorescence in the ganglion cells, inner nuclear (nuclei of amacrine, bipolar, and horizontal cells), and outer nuclear (nuclei of photoreceptors) layers, suggesting that all neural cells of the retina are accessible by PS-ODNs following intravitreal injection. Injection of fluorescent molecule 6-FAM demonstrated rapid clearance from the vitreous and no existence in the neuroretina as early as 24 hours following injection, indicating that the fluorescence observed in PS-ODN injected eyes was associated with the fluorescently labelled PS-ODN itself. The access of PS-ODNs to the retina may be of significance in attenuating neural degeneration in several retinal diseases. Neural damage is a key event in glaucoma and ischaemia initiated retinal degeneration, which have recently been thought to be affected by excessive stimulation of receptors such as N-methyl-D-aspartate receptor and aberrant expression of pathogenic genes.[9 10 15 16] It has been documented that ODNs against pathogenic genes are effective in preventing neuronal degeneration in the brain.[2–4] The therapeutic effect of ODNs was also demonstrated in cancer and virus infectious diseases including virus retinitis.[1 5 17 25] Based on these facts, the access of PS-ODNs to the neural retina may be of significance in attenuating retinal degeneration by targeting pathological genes.

The use of Genescan analysis allowed us to demonstrate that PS-ODNs remained detectable in the retina for at least 12 weeks with only the minor appearance of shortened degradation products. Such products result from 3' exonuclease activity since they remain associated with the fluorescent marker. Degradation from the 5' end of the ODN is not detected by this technique as this involves cleavage from the 6-FAM molecule. The presence of fluorescent ODN even at 12 weeks after injection would suggest that while this mechanism may operate in vivo, it is not markedly significant in the process. This experiment, along with our previous results,[19] demonstrates the marked stability of PS-ODNs in the retina. This is in contrast with a recent report which suggested that PS-ODNs were eliminated from the murine retina within 3 days of injection.[20] Our preliminary results indicated that fixation of the retina by paraformaldehyde and glutaraldehyde significantly affects fluorescent signal detection (unpublished data). Using solid phase extraction followed by capillary gel electrophoresis, Leeds and her colleagues[21 22] demonstrated that PS-ODNs remained detectable in the retina for 14 and 20 days after injection in monkey and rabbit eyes respectively. These differences may reflect species variation, the significantly lower concentrations administrated in the murine,[20] monkey,[21] and rabbit[22] eyes, and the different sensitivity of detection methods.

Following intravitreal injection, PS-ODNs preferentially accumulated in the RPE cells. Histological examination indicated that PS-ODNs were localised in the cytoplasm of RPE (Fig 1C). PS-ODNs have been supposed to be internalised by the process of adsorptive endocytosis and fluid phase pinocytosis mediated by cell surface integrins.[1 29] It has been demonstrated that RPE cells require the integrin receptor $\alpha_v\beta_5$ specifically for the binding of photoreceptor outer segments (POS), and integrin $\alpha_v\beta_5$ is thought to be a specific RPE receptor for phagocytosis.[30 31] We postulate that the PS-ODNs are originally taken up by the RPE cells through phagocytosis mediated by cell surface receptors such as integrin $\alpha_v\beta_5$, then released from the phagosomes into the cytoplasm after unsuccessful digestion owing to their resistance to nucleases.[22]

The preferential accumulation of PS-ODNs in the RPE may be of significance in investigating the molecular mechanism of retinal degeneration in which the main cause of photoreceptor loss is assumed to be related to RPE dysfunction, such as ARMD.[13 14 32] The RPE is a single cell layer situated between the neural retina and the choroid. Among the roles performed by the RPE, phagocytosis is the vital function which results in complete turnover of the shed POS. There is a continuous build up of photoreceptor derived debris (lipofuscin) in the RPE with age.[32] Progressive engorgement of RPE cells with these functionless residues is associated with the extrusion of aberrant materials which accumulate in Bruch's membrane and aggregate in the form of drusen and basal laminar deposits (BLD).[13 14] These excretions contribute to the further deterioration of the RPE which eventually leads to the visual cell death.[33] At present the molecular mechanism of the formation of drusen and BLD in ARMD is unclear. The process of POS through the RPE cells involves POS binding to the RPE surface, subsequent internalisation, and digestion by a series of lysosomal enzymes.[32] Using antisense strategies, we have previously demonstrated that downregulation of lysosomal enzymes by targeting appropriate genes, such as cathepsin D and S, induces POS derived debris accumulation in the RPE cells.[34–37] As the variety of cellular mechanism of POS processing in the RPE are continuously revealed, PS-ODNs can be used as an effective tool to investigate the target genes which may exert a trigger impact on the progressive photoreceptor loss related to RPE dysfunction.

Ophthalmic laser treatment is a commonly used therapy for many retinal diseases. The efficacy of laser photocoagulation for the treatment of macular CNV in patients with ARMD and retinal neovascularisation in patients with diabetic retinopathy has been documented.[38 39] However, there is non-selective destruction of healthy retinal elements by the laser beam and sometimes by delayed expansion of the laser induced destructive effects to adjacent retina.[40] This is particularly important for laser application adjacent to the central foveal area. A

recent study indicates that glutamate, the main excitatory neurotransmitter of photoreceptor neurons, is involved in the pathogenesis of laser induced retinal neural injury.[12] In the present study, we have demonstrated that PS-ODNs are strongly distributed in laser treated regions for a prolonged period (at least 8 weeks after photocoagulation). As ODNs have demonstrated their protective effects in neuronal injury induced by excitatory amino acids,[12 41] an effective retinal neuroprotection may be clinically achieved in patients following laser photocoagulation by blocking gene expression of the excitatory receptors such as N-methyl-D-aspartate receptor.[12]

Loss of vision in the late phase of ARMD results from the effects of leakage and subretinal haemorrhage due to neovascular membranes.[13 14] The protocol of photocoagulation employed in the present study has been confirmed to produce CNV which resembles neovascular membranes in ARMD.[23] We have demonstrated that injected PS-ODNs preferentially accumulate at the site of laser photocoagulation for at least 8 weeks, predominantly localised to the RPE, local choroidal tissue, and infiltrating cells recruited by the injury response. The infiltrating cells were further confirmed to be macrophages. Macrophages, RPE, and choroidal fibroblasts have been considered to be the main sources of angiogenic factors in experimental CNV induced by intense laser photocoagulation,[23 42 43] and in neovascular membranes of the eyes with ARMD.[44 45] The effectiveness of ODNs in inhibiting neovascularisation by blocking angiogenic gene expression has been documented.[17 18] Interruption of angiogenic gene expression using PS-ODNs to inhibit the onset of neovascularisation may be an effective point for therapeutic intervention of CNV. As expected, the random and anti-VEGF specific PS-ODNs showed no differences in distribution and persistence in the normal retina following intravitreal injection. The next step therefore will be to examine the functional effect of the anti VEGF specific PS-ODN on experimental CNV formation.

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